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Carboxymethylation of a Ribonuclease from *Rhizopus* sp.

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In order to investigate the nature of the amino acid residues involved in the active site of a ribonuclease from *Rhizopus* sp. (RNase Rh), carboxymethylation of RNase Rh with iodoacetate was performed.

RNase Rh was found to be inactivated markedly at pH 3—5 by iodoacetate. From the pH profile of the rate of inactivation of RNase Rh, it was suggested that functional groups having pK_a values of *ca.* 7.3 and 4.3 might be involved in this inactivation reaction.

The determination of the amino acid composition of RNase Rh inactivated by iodoacetate at pH 5.0 indicated that the formation of about one residue of N^3-carboxymethylhistidine was responsible for the loss of enzymatic activity. The results were very similar to those for an RNase from *Asp. saitoi* having very similar base specificity.

The carboxymethylation of RNase Rh was inhibited by competitive inhibitors. Thus, the histidine residue modified might be involved in the active site of the enzyme.

Keywords——Ribonuclease; *Rhizopus*; chemical modification; histidine; ribonuclease; active site

The ribonuclease isolated from *Rizopus* sp. (RNase Rh) by Tomoyeda *et al.* is base non-specific and its molecular weight was determined to be 24000.[1] In previous papers, we suggested from kinetic evidence[2] and a chemical modification study[3] that a histidine residue having pK_a 7.0 was involved in the active site of the RNase Rh.

The alkylation of RNase Rh by iodoacetamide inactivated RNase Rh with concomitant formation of 2 mol of carboxamidomethylated histidine residues.[4] The results indicate that at least one histidine residue is involved in the active site of this enzyme.

In this study, in order to investigate the structure–function relation of RNase Rh further, alkylation of RNase Rh with iodoacetic acid was carried out, since alkylation has been very useful for probing the active sites of other ribonucleases (RNases).[5–10] By quantitative analysis of the alkylated amino acid residues in RNase Rh after reaction with iodoacetic acid, it was found that about one histidine residue is modified preferentially during inactivation. Further, the location of the carboxymethyl group introduced was determined to be the N^3-position of a histidine residue in RNase Rh.

Materials and Methods

Enzyme——RNase Rh used in this study was purified from a commercial digestive "Gluczyme" (*Rhizopus* sp.) according to the method reported previously.[11]

Chemicals——Iodoacetic acid was obtained from E. Merck. Iodo[2-^{14}C]acetic acid (57 mCi/mmol) was purchased from the Radiochemical Centre, Amersham. Ribonucleic acid (RNA), 2′-cytidine monophosphate (2′-CMP) and 2′(3′)-uridine monophosphate (2′(3′)-UMP) were obtained from Kojin Co. 2′-adenosine monophosphate (2′-AMP) and 2′-guanosine monophosphate (2′-GMP) were purchased from Sigma Chem. Co. (St. Louis, Mo., U.S.A.). Carboxymethyl derivatives of histidine and methionine were prepared according to the procedures of Goren *et al.*[12] with some minor modifications.

Assay of RNase Activity——The enzymatic activity was measured principally according to the method reported by Komiyama and Irie.[11] The reaction mixture (2 ml), containing 5 mg of RNA in 0.05 M acetate buffer (pH 5.0) and the enzyme, was incubated at 37 °C for 5 min, then 1 ml of MacFadyen's reagent[13] was added to stop the reaction. The precipitate was removed by centrifugation and 0.3 ml of the supernatant was diluted with 2 ml of water. The absorbancy of the solution at 260 nm was determined.

Protein Concentration——Protein concentration was determined spectrophotometrically assuming the absorbancy at 280 nm of 0.1% RNase Rh to be 1.91.[14]

Carboxymethylation of RNase Rh——RNase Rh (1.78 μM) was incubated with iodoacetic acid (0.1 M) in 0.1 M acetate buffer, at 37 °C. Enzyme inactivated to an appropriate extent was dialyzed against distilled water with several changes of the outer solution (5 l) for 3 d. Radioactive iodoacetic acid used for the incorporation experiments had a radioactivity of 2 μCi/mg.

The rates of inactivation of RNase Rh at various pH's were measured as described above using 0.1 M acetate buffer (pH 3.0—6.0) and 0.1 M Tris-HCl buffer (pH 7.0—9.0). The enzyme concentration used for this experiment was 3.6 μM.

Amino Acid Analysis——A solution of native RNase Rh or carboxymethylated RNase Rh (CM-RNase Rh, 0.2—0.3 mg) was evaporated to dryness *in vacuo* over P_2O_5 and hydrolyzed in 6 N HCl in an evacuated, sealed tube for 24 h at 110 °C. The amino acid composition of the hydrolyzate was determined by the method of Spackman *et al.*[15] and analyzed with a Nihondenshi JEOL 6AH amino acid analyzer.

Performic acid oxidation was performed according to Hirs' procedure.[16]

Circular Dichroism (CD) Spectrum——CD spectra were measured with a JASCO J40 spectropolarimeter. The light path of the cell used was 0.05 cm. The protein concentration used was *ca.* 0.0034 μM. All data are expressed as molar ellipticity.

Ultraviolet Difference Spectophotometry——All measurements were conducted at 22 °C using tandem cells having a 0.44 cm light path with a Hitachi 100—50 recording spectrophotometer.

High-Voltage Paper Electrophoresis——High-voltage paper electrophoresis was carried out using pyridine–acetate buffer, pH 6.5 (pyridine : acetic acid : water = 25 : 1 : 225, v/v) at a voltage of 200 V/cm for 120 min. The radioactivity on the paper electrophoresis strip was measured in each 5 mm section of paper in the scintillant described below.

Radioactivity Counting——Radioactivity measurements were carried out with an Aloka liquid scintillation spectrometer, model LSC-602. For bottle counting, about 100 μl of sample was added to a vial containing 0.3 ml of 1 M hyamine chloride in ethanol and 10 ml of the scintillation liquid (4 g of 2,5-diphenyloxazole and 100 mg of *p*-bis[2′-(5′-phenyloxazolyl)]benzene in 1 l of toluene).

Results

pH Dependence of the Inactivation of RNase Rh by Iodoacetic Acid

The rates of inactivation of RNase Rh by iodoacetic acid were measured at various pH values. The results are shown in Fig. 1. The rate of inactivation was maximum at pH 4.0, and it decreased markedly above pH 8.0.

Alkylation of RNase Rh by Iodoacetic Acid

Although the optimal pH for the inactivation of RNase Rh with iodoacetate was at pH 4.0, in view of the acid stability and enzyme solubility,[4] the alkylation of RNase Rh with iodo[2-^{14}C]acetic acid was performed mostly at pH 5.0 and 37 °C. The rate of incorporation of radioactive carboxymethyl group was measured. The relation between the amount of carboxymethyl group incorporated and the remaining enzymatic activity is shown in Fig. 2. The extrapolation of the curve to zero activity indicated that the incorporation of about 1.2—1.3 carboxymethyl groups was related to the loss of enzymatic activity.

The amino acid composition of RNase Rh inactivated to 30% of the original activity is shown in Table I. Carboxymethylation with iodoacetic acid caused the loss of about 0.75 mol of histidine residues. The amounts of amino acid residues other than those listed in Table I were similar to those in native RNase Rh.

CD Spectra of RNase Rh and CM-RNase Rh

In order to investigate the conformation of CM-RNase Rh, the CD spectrum of RNase Rh was measured in the wavelength region of 200—260 nm. The CD spectrum of RNase Rh

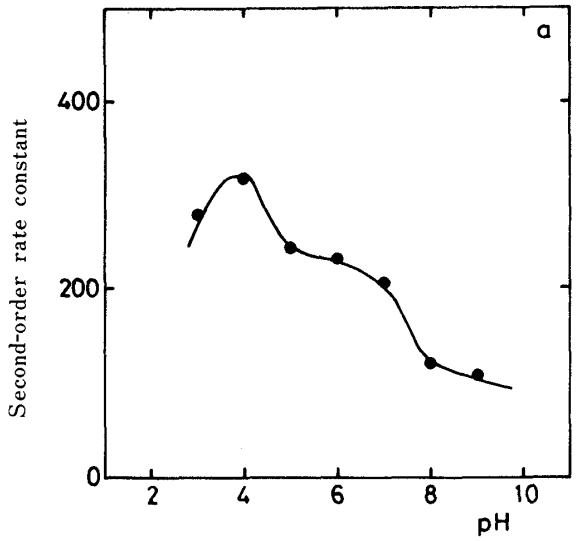

Fig. 1a. Effect of pH on the Inactivation of RNase Rh with Iodoacetic Acid

The enzyme (1.78 μM) was incubated with iodoacetic acid at various pH's at 37 °C. The rates of inactivation were measured as described in Materials and Methods. The concentration of the reagent was 0.1 M throughout this experiment.

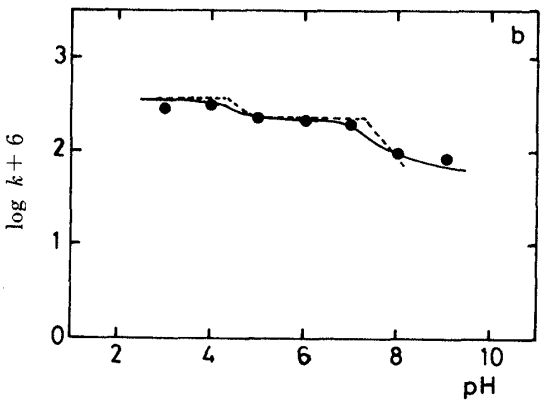

Fig. 1b. pH Profiles of log k of Inactivation of RNase Rh with Iodoacetic Acid

The data are replotted from Fig. 1a.

in the shorter-wavelength region (between 200 and 240 nm), which reflects the protein backbone structure, showed a trough at 205 nm and a negative shoulder at 215 nm (Fig. 3). The CD spectra of CM-RNase Rh's having remaining activity of up to 32% were practically identical with that of the native enzyme.

The results indicated that, in the early stage of carboxymethylation, RNase Rh retained the conformation of the native enzyme as far as measured by CD spectroscopy.

Effect of Nucleotide on the Inactivation of RNase Rh by Iodoacetic Acid

When alkylation by iodoacetic acid at pH 5.0 was performed in the presence of a competitive inhibitor, such as 2′-AMP, 2′-GMP, 2′-CMP, or 2′(3′)-UMP, the rate of inactivation decreased, though the extent of the decrease depended on the competitive inhibitor used (Fig. 4).

The extents of protection of RNase Rh from inactivation by these competitive inhibitors were parallel to their inhibitory effects on the enzymatic activity.[2] These data suggest that the amino acid residues modified by iodoacetic acid may be involved in the active site of RNase Rh or at least located near the active site.

Difference Spectrum of CM-RNase Rh with 2′-AMP

The interaction of CM-RNase Rh with the most potent inhibitor, 2′-AMP, was examined spectrophotometrically. The difference spectra of CM-RNase Rh and the native RNase Rh induced by addition of 2′-AMP are shown in Fig. 5.

The trough of the spectrum appeared at 255 nm, which is very similar to that of a RNase from *Asp. saitoi* with 2′-AMP.[8] Although the CM-RNase Rh showed weaker binding with 2′-AMP than that of native enzyme, the binding ability to 2′-AMP still remained at this stage of modification.

Based on these results, the deviation of the optical density at 255 nm of enzyme–2′-AMP mixture from the sum of those of each component was plotted at various 2′-AMP concentrations. According to Hummel's equation,[17] the dissociation constant of the enzyme–inhibitor complex was calculated. The binding constants for the native RNase Rh and CM-

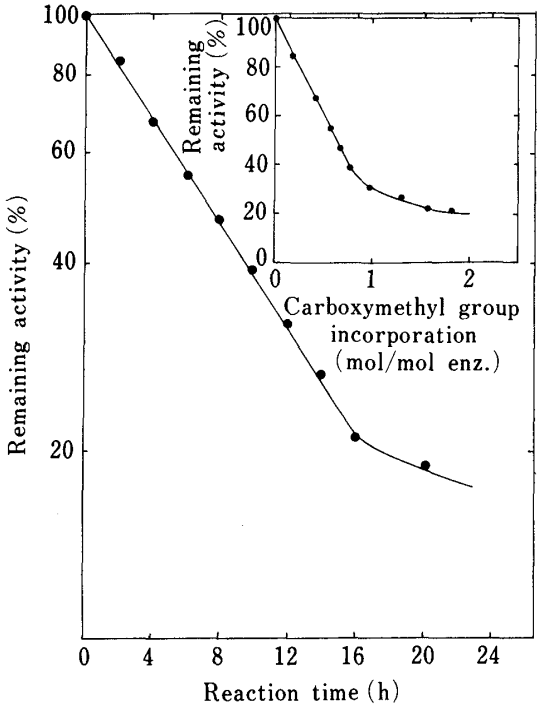

Fig. 2. Time Course of Carboxymethylation of RNase Rh at pH 5.0

RNase Rh (6 μM) was incubated in 6 ml of 0.1 M acetate buffer, pH 5.0 containing 15 μCi of iodo[2-^{14}C]acetic acid at 0.1 M concentration. The reaction was carried out at 37 °C. The radioactivity was measured as described in Materials and Methods.
Insert: Relationship between enzymatic activity and carboxymethyl groups incorporated into RNase Rh.

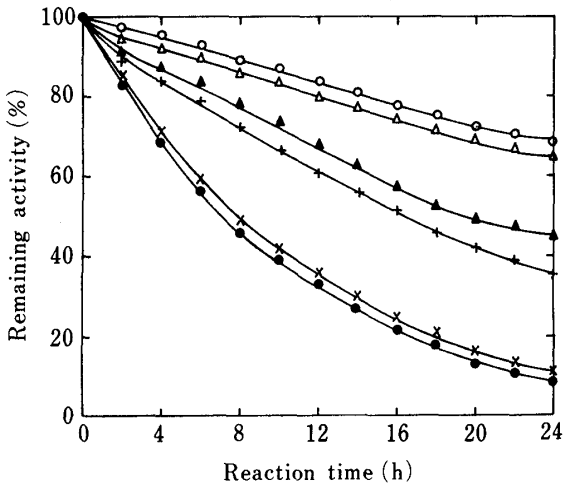

Fig. 4. Effects of Nucleotides and Adenosine on the Inactivation of RNase Rh with Iodoacetic Acid

The enzyme (1.78 μM) was incubated with iodoacetic acid (0.1 M) in the presence of 20 mM inhibitor in acetate buffer (0.1 M, pH 5.0) in the dark at 37 °C. The enzymatic activity was measured by determining acid-soluble nucleotides after enzymatic digestion of RNA. ○, 2′-AMP; △, 2′-GMP; ▲, 2′-CMP; +, 2′(3′)-UMP; ×, adenosine; ●, no addition.

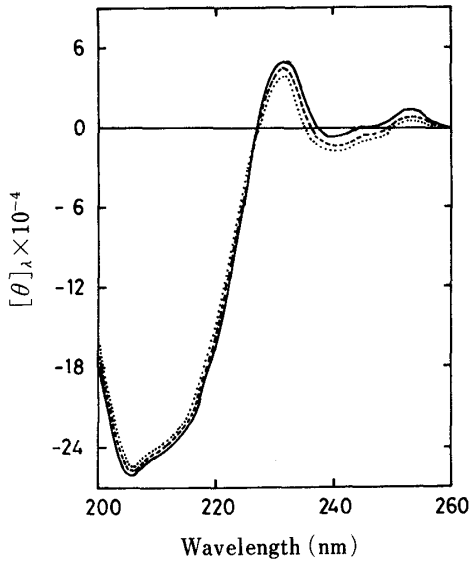

Fig. 3. CD Spectrum of RNase Rh Carboxymethylated with Iodoacetic Acid at pH 5.0 (0.1 M, Acetate Buffer)

The experimental conditions were as described in the text. Native RNase Rh (——); CM-RNase Rh having 65% activity (- - - -); and that having 32% activity (------). The enzyme concentration was 0.0034 μM.

TABLE I. Amino Acid Compositions of Carboxymethylated and Native RNase Rh

Amino acid	Calculated No. of amino acid residues[a]		Theoretical No. of amino acid residues of RNase Rh[b]
	CM-RNase Rh	Native RNase Rh	
Lysine	9.74	10.16	9
Histidine	3.18	3.83	4
Arginine	4.00	4.00	4
Methionine[c]	4.25	4.47	4

a) Numbers of amino acid residues were calculated from amino acid analysis of 24-h hydrolysates, assuming the amount of arginine to be 4 residues. *b*) Taken from reference 11. *c*) Obtained from an alkaline hydrolysate.

TABLE II. The Binding Constants of Native RNase Rh and CM-RNase Rh with 2′-AMP at pH 5.0[a]

Enzymatic activity (%)	K_i[b] (μM)
100	18.95
39.5	37.28

a) The alkylation conditions were as described in the text and Fig. 2. *b*) The binding constants of native RNase Rh and CM-RNase Rh were determined as described in Materials and Methods.

RNase Rh are shown in Table II. The binding constant for the modified enzyme having *ca.* 39.5% activity was about twice as large as that of the native RNase Rh. The decrease in the binding ability might be due to a change of the conformation of the active site or to blocking of the binding with 2′-AMP by the modification.

Identification of Carboxymethylated Amino Acid Residues in RNase Rh Modified by Iodo[2-^{14}C]acetic Acid

In order to identify the amino acid residues carboxymethylated at pH 5.0, ^{14}C-CM-RNase Rh's having 72, 55, 40 and 25% activities were hydrolyzed with 6 N HCl in evacuated, sealed tubes at 100 °C for 24 h.

Each hydrolysate was subjected to high-voltage paper electrophoresis. A typical distribution of radioactivity on the paper electrophoresis strip is shown in Fig. 6. By this procedure, the radioactivity was separated into three fractions: an acidic fraction (including N^1,N^3-dicarboxymethylhistidine (di-CM-His) and *S*-carboxymethylhomocysteine (*S*-CM-homocysteine)), N^3-carboxymethylhistidine (3-CM-His), and the fraction located in the neutral amino acid zone. The last fraction may contain N^1-carboxymethylhistidine (1-CM-His) and carboxymethyl sulfonium methionine. The percentage of the radioactivity corresponding to each fraction was calculated and the results are shown in Table III.

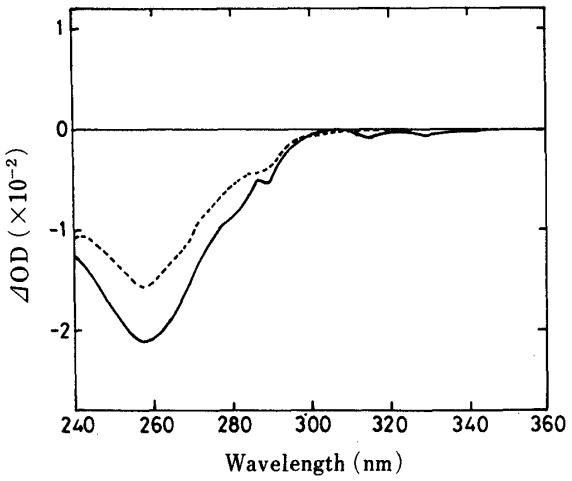

Fig. 5. Difference Spectra Induced by Addition of 2′-AMP to RNase Rh and CM-RNase Rh at pH 5.0

The experimental conditions were as described in Materials and Methods. The native RNase Rh, (——); CM-RNase Rh having 25% activity, (– – – –).

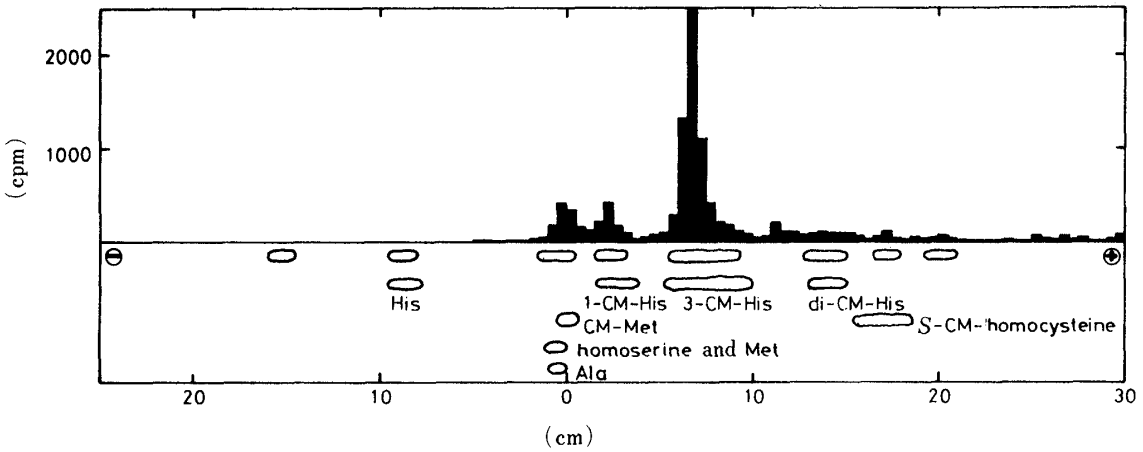

Fig. 6. Identification of the Amino Acid Residues of RNase Rh Modified by Iodo[2-^{14}C]acetic Acid at pH 5.0

CM-RNase Rh having about 25% residual activity was hydrolyzed as described in Materials and Methods. The hydrolysate was separated by paper electrophoresis at pH 6.5. The radioactivity was measured as described in the text. The positions of standard amino acid derivatives were visualized by spraying 0.2% ninhydrin in acetone.

TABLE III. Determination of Carboxymethylated Amino Acids in CM-RNase Rh

Sample	Reaction time (h)	Residual activity (%)	1-CM-His	3-CM-His	di-CM-His	CM-Met	Unknown
			(mol/mol enzyme)[a]				
1	5	72	0.056	0.335	0.017	0.045	0.144
2	8	55	0.042	0.455	0.034	0.041	0.176
3	14	40	0.088	0.690	0.039	0.066	0.192
4	17	25	0.176	1.145	0.097	0.056	0.295

a) Since the recovery of amino acid derivatives was between 93—98%, no correction for the recovery of each amino acid during the elution process was made in this experiment. The abbreviations used in the table is: CM-Met, carboxymethylmethionine sulfonium salt.

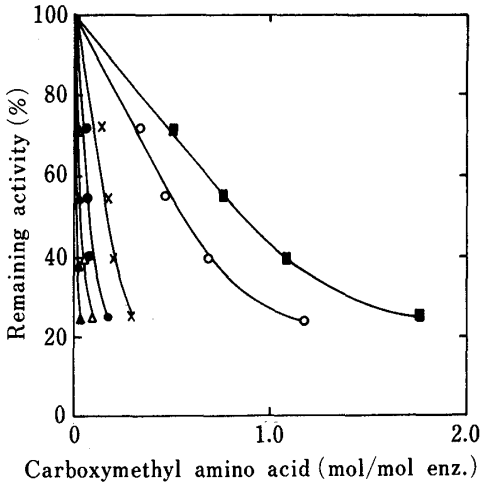

Fig. 7. Relationship between the Enzymatic Activity and Carboxymethyl Groups Incorporated into RNase Rh

The figure was replotted from data listed in Table III. ○, 3-CM-His; ●, 1-CM-His; △, di-CM-His; ▲, CM-Met; ×, unknown; ■, total CM-amino acid.

The relation between the enzyme activity and the amount of each amino acid residue alkylated with a carboxymethyl group in RNase Rh is shown in Fig. 7. The incorporation of carboxymethyl groups into each amino acid proceeded linearly in the early stage of the reaction, but the curve deviated from linearity when the residual activity fell below 40%. The incorporation of carboxymethyl group into methionine and the formation of 1-CM-His and di-CM-His were too small to account for the loss of enzymatic activity.

In the early stages of the reaction, the formation of 3-CM-His seemed to be linearly correlated with the loss of enzymatic activity. When the curve was extrapolated to zero activity, the incorporation of carboxymethyl group into N^3-histidine was about one residue per mol of enzyme. Thus, it may be concluded that the cause of inactivation of the enzyme by carboxymethylation is the formation of about one N^3-carboxymethylhistidine residue.

Discussion

RNases are easily inactivated by halofatty acids in general. Typical examples were reviewed by Richards and Wyckoff[18] and Takahashi and Moore.[19] However, the site of carboxymethylation and the rate of inactivation differ depending on the nature of each RNase. The pseudo-first order rate constant of the modification of RNase Rh by iodoacetate was about 1/20 of those of RNase A[20] and RNase T_1,[21] but was very similar to that of an RNase from *Asp. saitoi*.[8]

Since the RNase Rh was protected against alkylation by competitive inhibitors and the protective effects of the nucleotides paralleled the inhibitory effect of the nucleotides,[2] it may

be concluded that the site of carboxymethylation is within the active site of RNase Rh.

The identification of the site of carboxymethylation was performed by paper electrophoresis, after acid hydrolysis. The major ^{14}C-labeled compound formed was 3-CM-His. The site of incorporation of the carboxymethyl group into RNases seems to very closely related to the base specificity of the enzyme. For RNase A, which is specific for pyrimidine bases, the carboxymethyl group was incorporated mostly into the N^1-position of histidine residue.[20] In the cases of guanine-specific or guanine-preferential RNases, the incorporation of carboxymethyl groups occurs mostly at the carboxyl group of glutamic acid.[7,9-11] The formation of 3-CM-His observed in this work is consistent with that of an RNase from *Asp. saitoi* which has a base specificity very similar to that of RNase Rh. Therefore, judging from two experiments carried out on base non-specific and adenine-preferential RNases, the formation of 3-CM-His may be a common phenomenon in such RNases in general, though more data are required to confirm this.

The pH-dependence of the rate of inactivation of RNase Rh by iodoacetate is plotted in the form of $\log k$–pH in Fig. 1b. The guide lines drawn according to Dixon's theory[22] (shown in Fig. 1b) suggest that two functional groups having pK_a values around 7.3 and 4.3 are related to the inactivation of RNase Rh. The former value is very similar to that of one of the functionally essential histidine residues previously identified.[3] The small inflection observed at pH 4.3 also suggested the contribution of a carboxyl group to the inactivation reaction, and thus to the active site of RNase Rh, since we have already reported, based on the modification of RNase Rh by a water-soluble carbodiimide,[23] that a carboxyl group is essential in the active site. However, whether the pK_a of this carboxyl group is 4.3 or not should be clarified by further experiments.

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